APPENDIX C

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Atty. Docket: YARKONI2

In re Application of:	)	Conf. No.: 1591
Shai YARKONI et al	)	Art Unit: 1646
Appln. No.: 09/147,346	)	Examiner: L. Helms
Filed: March 1, 1999	)	
For: CHIMERIC TOXINS FOR TARGETED THERAPY	)	

DECLARATION UNDER 37 C.F.R. §1.132

Honorable Commissioner for Patents
U.S. Patent and Trademark Office
2011 South Clark Place
Customer Window, Mail Stop
Crystal Plaza Two, Lobby, Room 1B03
Arlington, VA 22202

Sir:

I, Haya Lorberboum-Galski hereby declare and state as follows:

I am one of the co-inventors of the subject matter of the above referenced patent application. A true and correct copy of my *Curriculum Vitae* is attached hereto as Exhibit A.

I am the same Haya Lorberboum-Galski who previously executed a declaration in this case on June 11, 2003, which, on information and belief, was filed on June 16, 2003. As the gel of Figure 1 therein may not have been sufficiently clear,

and as the explanations therein may have been somewhat confusing, the present declaration is being filed containing the same experimental data as in my previous declaration but adding another experiment (Figure 2) and further clarifying the points I had intended to make. It is, therefore, my intention that the present declaration supersede my previous declaration.

I am thoroughly familiar with the contents of the above referenced patent application.

I am thoroughly familiar with the contents of all of the references that have been cited in support of rejections of the patentability of the claims of the above referenced patent application.

I have conducted, or have had conducted under my supervision and direction, all of the tests discussed below.

The Met-GnRH-PE of the present invention has a structure that is different from that of the GnRH used by Nett et al in U.S. patent 5,378,688. Furthermore, it has unexpectedly been discovered that this difference in structure allows a significant difference in selectivity and specificity of the two targeting molecules.

It can be seen in the experiments on pages 10 and 11 of the present specification (as originally filed), in conjunction with Figure 1C, that the plasmid used, when

expressed in a bacterial expression system, will result in a polypeptide in which the upstream GnRH sequence is preceded by a Met residue. As such a preceding Met residue will be present regardless of whether the natural human GnRH sequence is used or an analog thereof and regardless of the sequence of the toxic portion of the polypeptide, the present declaration will always refer to the chimeric protein of the present invention as Met-GnRH-cell killing moiety, e.g., Met-GnRH-PE66, Met-GnRH-PE40 or Met-GnRH-DFF40. In order to clarify this difference between the chimeric protein of the present invention and the conjugates of the prior art, the present specification has, on information and belief, now been amended to also refer to the targeting portion of the chimeric proteins of the present invention as Met-GnRH.

The targeting unit used in the conjugates of Nett is not bacterially produced and does not have a preceding Met. Thus, it is appropriate to refer to the products of Nett as GnRH conjugates, as opposed to the Met-GnRH chimeric proteins of the present invention.

Nett discloses that the GnRH used for targeting in its invention targets the pituitary gland (see column 12, lines 34-38 of Nett). Indeed, Nett states that the gonadotropin secreting cells of the anterior pituitary gland

are the only cells to which the gonadotropin-releasing hormone portion of Nett's conjugates will bind.

In contrast, the Met-GnRH of the present invention can selectively target cells having a distinctly different GnRH binding site, which appears, for example, on adenocarcinomas.

To clarify which of the cell lines used in the experimentation in the present specification are adenocarcinomas, the present specification has, on information and belief, now been amended to change the term "carcinoma" to read "adenocarcinoma" for each of those cell lines that are inherently adenocarcinomas. Thus, Table 1 of the present specification at page 13 (as originally filed) refers to the Caco2, HT-29, SW-48, OVCAR3 and MDA MB-231 cell lines as being carcinomas. However, it was well known as of the filing date of the present application that each of these cell lines are, indeed, adenocarcinomas. Attached hereto as Exhibit B are pages 124, 222, 226, 227 and 272 from the ATCC Catalog of Cell Lines and Hybridomas (7^{th} Edition, 1992), listing each of these cell lines. The above-listed cell lines, respectively ATCC HTB 37, ATCC HTB 38, ATCC CCL 231, ATCC HTB 161 and ATCC HTB 26, are all identified in this 1992 catalog as adenocarcinoma cell lines. Accordingly, as all of these cell lines are inherently adenocarcinomas, the specific identification of

them as such in the present specification is not new subject matter, but only makes explicit what had been inherent and a matter of common knowledge in the art.

While the HepG2 cell line has many of the attributes of an adenocarcinoma, because of the unique characteristics of liver cells, it has been given the specific characteristic name hepatocellular carcinoma, or hepatocarcinoma.

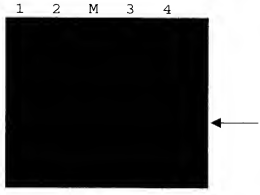
With respect to the primary cultures, I can state of my own personal knowledge that all of the carcinomas referred to in Experiment 5 of the present specification were independently classified as adenocarcinomas by a pathologist. See Nechushtan et al, "Adenocarcinoma cells are targeted by the new GnRH-PE_{66} chimeric toxin through specific and gonadotropin-releasing hormone binding sites", J Biol Chem 272:11597-11603 (1997), at the sentence bridging pages 11600-11601. This publication of the invention disclosed in the present application is attached hereto as Exhibit C.

To show the selectivity of the Met-GnRH of the present invention and how it differs from that of the GnRH that is reported by Nett, the following experiment has been conducted. In order to determine the presence or absence of pituitary GnRH receptor in various cell lines, PCR analysis was conducted. The mRNA obtained from the cell lines was converted to template-cDNA and subjected to PCR reaction. The

primers used for the PCR reaction were 5-GCTTGAAGCTCTGTCCTG GGA-3 (sense starting from nucleotide -25, according to Kakar et al, Biochem Biophys Res Comm, 189:289-295 (1992)) and 5-GATAAGTGGATCAAAGCATGG-3 (antisense starting from nucleotide 946). The reaction mixture was incubated in a DNA thermal cycler (MJ Research, Inc, USA) for 39 cycles. Each cycle consisted of 1 minute at 94°C, 1 minute at 60°C and 2 minutes at 72°C.

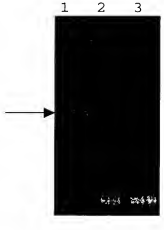
The following types of cells were subjected to this PCR reaction: normal human lymphocytes, granulosa cell tumor cells, normal human granulosa cells, normal human pituitary cells, and the Caco2 adenocarcinoma cell line. The results are shown in the following Figures 1 and 2.

Figure 1



Lane 1: template-cDNA prepared from normal human lymphocytes
Lane 2: template-cDNA prepared from granulosa cell tumor
Lane M: marker.
Lane 3: template-cDNA prepared from normal human granulosa cells
Lane 4: template-cDNA prepared from normal human pituitary cells
Arrow indicates the expected PCR product of 991 bp

Figure 2



Lane 1: template-cDNA prepared from normal human pituitary
Lane 2: template-cDNA prepared from Caco2 adenocarcinoma cells
Lane 3: template-cDNA prepared from Caco2 adenocarcinoma cells
Arrow indicates the expected PCR product of 991 bp

For each template cDNA, a housekeeping gene (such as β-actin) was checked to test for its quality. A PCR product

in the expected size was obtained for samples 1-3 above (data not shown).

It can be seen that the 991 bp pituitary GnRH receptor appears on granulosa cell tumor cells and normal human pituitary cells, but does not appear on normal human granulosa cells or Caco2 adenocarcinoma cells.

Next, we tested the effect of the Met-GnRH-PE66 of the present invention on cells containing the pituitary GnRH receptor and on cells that do not carry this receptor but do carry the GnRH binding site targeted by the present molecules.

Cells (10^4 in 0.2 ml culture medium) were seeded in 96-well microplates, and 20 hours later various concentrations of GnRH-PE66 were added. After an additional 24 hours incubation, [^{3}H]leucine (1 μCi (37 kBq)/well) was added overnight. The plates were then stored at -70°C for several hours, followed by quick thawing at 37°C. The cells were harvested on filters and the incorporated radioactivity was measured in a β counter. The results, shown in Table 1, are expressed as the percentage of protein synthesis of control cells not exposed to chimeric proteins.

Table 1

	Protein Synthesis, % of the Control 10 (ng Protein)	Protein Synthesis, % of the Control 50 (ng Protein)	Protein Synthesis, % of the Control 500 (ng Protein)
Granulosa Cell Tumor	102	99	104
Caco2	42	6	1

These data clearly show that the chimeric protein of the present invention does not kill granulosa cell tumor cells, which carry the pituitary GnRH receptor, but do target and kill Caco2 cells that do not carry the pituitary GnRH receptor but do carry a different GnRH binding site.

As is evidenced by the above data, the mechanism of action of the GnRH-toxin conjugate of Nett, which has been disclosed as being useful for treatment of hormone-dependent types of cancers, is completely different from the mechanism of action of the presently claimed chimeric protein. The GnRH-toxin conjugate of Nett is not proposed to work directly on the cancer cells but rather it has been proposed that it works by an indirect effect. The paragraph at column 12, lines 34-57, states that the "conjugates are specifically targeted to the gonadotropin-secreting cells of the anterior pituitary gland." Furthermore, Nett explicitly states that these pituitary cells are the only cells to which the gonadotropin-releasing hormone portion of applicant's

conjugate "will bind". By this directed attack on the pituitary cells, the gonads atrophy and are not able to secrete LH and FSH and thus are rendered sterile. As stated in the following paragraph of Nett, the inhibition of growth of sex steroid-dependent tumors is due to lack of secretion of steroid hormones in a sterilized animal/human carrying such tumors. Thus, the conjugates of Nett do not directly attack sex-hormone related tumors, such as cancer of the breast or prostate.

On the other hand, the Met-GnRH-PE chimeric protein of the present invention works through its direct action on the tumor cells bearing the GnRH binding sites targeted by Met-GnRH. The cell killing results because adenocarcinomas and hepatocarcinomas express GnRH binding sites, and the Met-GnRH-PE chimeric proteins bind to these binding sites, thus allowing the internalization of the chimeric protein directly into the adenocarcinoma or hepatocarcinoma cells. Upon internalization into the cancer cells, the *Pseudomonas* exotoxin killing moiety inhibits protein synthesis (the natural activity of the toxin), which leads to the death of the cells. Thus, Met-GnRH-PE chimeric proteins directly act on and kill the cancer cells (adenocarcinomas and hepatocarcinomas).

The applications of the GnRH-toxin conjugates (prior art) and the Met-GnRH-PE chimeric proteins (claimed invention) vary.

The GnRH-toxin conjugates (prior art) have been suggested mainly for the sterilization of animals (veterinary medicine). In human medicine these reagents are suggested to be used for the following limited purposes: to control fertility (to achieve infertility effects), to treat sex steroid-dependent tumors, of which the only mentioned ones are breast and prostate cancers, and for the treatment of endometriosis.

As demonstrated by the instant reported data, the Met-GnRH-PE chimeric protein of this invention is a cytotoxic agent for a wide variety of cancers, most surprisingly, even for cancers originated in non-hormone dependent tissue such as colon adenocarcinoma, lung adenocarcinoma, renal adenocarcinoma, hepatocarcinoma, and more.

The main applications of the instant claimed chimeric protein are:

- malignant carcinoma, including non-hormone dependent cancer (a wide variety of adenocarcinomas and hepatocarcinomas); and
- benign tumors of the uterus and hyperplasia, including uterine leiomyoma, endometriomas, benign

prostate hyperplasia, breast polycystic disease and pituitary adenoma.

A wide variety of killing proteins were used in the testing of the chimeric proteins of this invention, including toxin proteins (such as the PE toxin) and human apoptotic proteins (such as proteins of the Bcl-2 family - Bax, Bak, Bik, or the DNase DFF40), and all (when fused to the Met-GnRH targeting moiety), irrespective of whether they were bacterial or human pro-apoptotic proteins, caused cell death of only adenocarcinoma and hepatocarcinoma cells. It is also pointed out that the various killing moieties used not only differ in their origin, but also in their size.

These reported data further highlight the fact that the Met-GnRH sequence is, indeed, responsible for targeting the various chimeric proteins to the specific intended target cells, and any protein fused to Met-GnRH in the form of the chimeric protein of this invention, will enter the cell via the GnRH-binding sites.

The following tables demonstrate the activity and specificity of another Met-GnRH-based chimeric protein Met-GnRH-DFF40, in which an identical Met-GnRH targeting moiety is fused to the human pro-apoptotic protein DNA Fragmentation Factor 40 (DFF40):

Table 2
Effect of Met-GnRH-DFF40 on Various Human Cell Lines

	Cell Lines	Cell Type	Live Cells (% of control)[a]
Target Cell Lines	293	Renal cell adenocarcinoma	42±13
	Colo 205	Colon adenocarcinoma	25±10
	DLD-1	Colon adenocarcinoma	34±6
	HCT-15	Colon adenocarcinoma	40±1
	SW-48	Colon adenocarcinoma	41±2
	MCF-7	Breast adenocarcinoma	41±11
	HepG2	Hepatocarcinoma	50±4
Non-Target Cell Lines	T24A	Bladder carcinoma	96±7
	J82	Bladder carcinoma	97±5
	A204	Rhabdomyosarcoma	99±1

[a] ± Indicate the standard deviation of 3-4 experiments, preformed using 2-3 different chimeric protein preparations. Partially purified GnRH-DFF40 preparations were used in all experiments. Controls received PBS in equal volumes.

Table 3
Effect of Various Chimeric Proteins on 293 Renal Carcinoma Cells

	Chimeric Protein	Target	Live Cells (% of control)
Positive Control	Met-GnRH-DFF40	Adenocarcinoma	42
	Met-L-GnRH-PE66	Adenocarcinoma	13[a]
	Met-GnRH-BIK	Adenocarcinoma	47
Negative Control	IL2-BAX	Activated B,T cells	97
	BPP-BAX	MBP-T cells	140
	Fc-BAX	Mast cells	99

[a] Met-L-GnRH-PE66 was tested using a highly purified protein (Ben-Yehudah et al, Med Oncol, 1:38-45, 1999 (copy attached)); the other chimeric proteins were only partially purified preparations.

In conclusion, the data presented herein clearly support the assertion that the chimeric fused proteins of the instant invention are distinctly different from the conjugated proteins of the prior art and that the instant chimeric proteins possess unusual, unexpected and unobvious properties as compared to the conjugated proteins of the prior art.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false, statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false

statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

April 30, 2004
Date

Lorberboum-Galski
Haya Lorberboum-Galski